Q-WOODCHIP

Diagnostics and risk management of plant health threats in wood chips for bio-energy

-an EUPHRESCO II project
total budget ~400.000 EUR

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University of Copenhagen

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Huge amounts of wood and bark for bio-energy is imported into EU

Consumption is estimated to be 8-10 million tonnes in EU in 2009
http://www.pellet.org/linked/2010-07-09%20wpac%20nb-doe.pdf

Quarantine pests and pathogens getting a free ride?

Scandinavian Journal of Forest Research, 2012; 27: 285–297

Taylor & Francis

ORIGINAL ARTICLE

Detection probability of forest pests in current inspection protocols – A case study of the bronze birch borer

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Challenge….

- The main conclusion of this work was:

- "As an example, we focused on the North American beetle *Agrilus anxius* (bronze birch borer) that can cause 100% mortality of European and Asian birch species in North America. **We simulated the process from logging in North America to sampling the wood chips upon arrival in Europe. The probability of pest detection for current sampling protocols used by port inspectors was very low (<0.00005), while a 90% chance of detection may require sampling 27 million litres of wood chips per shipload.**"

Q-Woodchip Consortium

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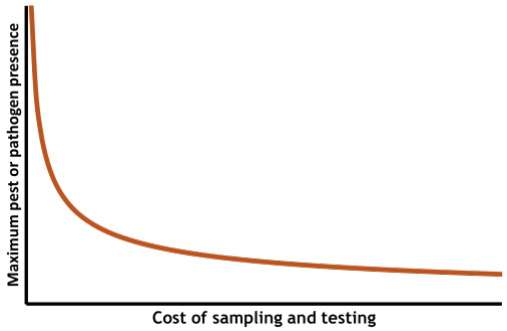
Diagnostics and risk management of plant health threats in wood chips for bio-energy

- Sampling strategies
- Priority list of pests and pathogens
- Recommendations for pre-export treatments for P&P reduction
- Detection and diagnosis of P&P
- Determination of tree species and geographical origin

Sampling - Tools for reducing risk

- How can we use modern analytical techniques to discriminate between high and low-risk lots of woodchips?
- Quantitatively: how much assurance can we provide about absence of pests and pathogens using sampling and testing and how much might it cost to achieve?



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A risk equation for woodchip lots

$$R = S.P_H.D_H.P_s = \frac{Q.P_s}{(1 - P_s)}$$

- R The risk associated with the lot; the number of surviving pests or quantity of viable pathogen
- S The total size of the lot
- P_H The proportion of lot formed by host wood
- Q Density of dead pests or non-viable pathogen in the lot
- D_H The density of pests or pathogens per mass of host in source
- P_S The probability that the pest of pathogen survives processing treatment and storage

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Options for reducing risk by testing

$$R = S.P_H.D_H.P_s = \frac{Q.P_s}{(1 - P_s)}$$

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Scenario: Emerald ash borer

$$R = S.P_H.D_H.P_s = \frac{Q.P_s}{(1-P_s)}$$

- R The risk associated with the lot: the number of surviving pests or quantity of viable pathogen
- S The total size of the lot: **21 505 T**
- P_H The proportion of lot formed by host wood: **0.3**
- Q Density of dead pests or non-viable pathogen in the lot
- D_H The density of P&Ps per mass of host in source: **19.0 T^{-1}**
- P_S The probability that the pest or pathogen survives processing treatment and storage: 0.00016

Adapted from Økland et al 2011

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Emerald ash borer: options for confirming low risk

$$R = S.P_H.D_H.P_s = \frac{Q.P_s}{(1-P_s)}$$

- R Sample 320 000 kg to examine for surviving pests
- Q Sample 20 - 200kg to test for the presence of pest DNA
- P_H Sample 2 - 13kg to test for host genera DNA

NGS method developed for wood genera

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$$L_D = 1 - \left(1 - \frac{1 - 0.05^{1/r}}{1 - f_N}\right)^{r/n}$$

- Lab sample limit of detection 1 – 10%: in principle replicate testing can reduce LOD in lot to any desired value

- BUT!
- Variation in different parts of lot may increase the LOD

- Test method applied to samples taken from woodchip lot

Alnus Unassigned Strobus Picea Quercus Carya Salix Fraxinus Pinus Acer Ulmus

Samubucus Cornus Ilex Betula Corylus Castanea Fagus Pterocarya Eucalyptus Aesculus Citrus Platinus Cedrus LarixPrunus arborea Prunus Sorbus Castanea Populus Tilia Acer pubinerve

Sources of sampling and test variation

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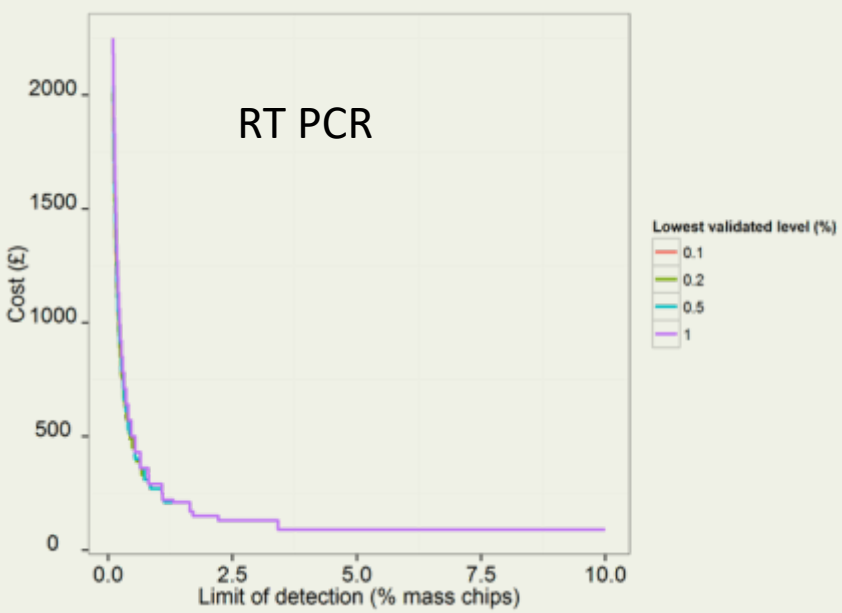
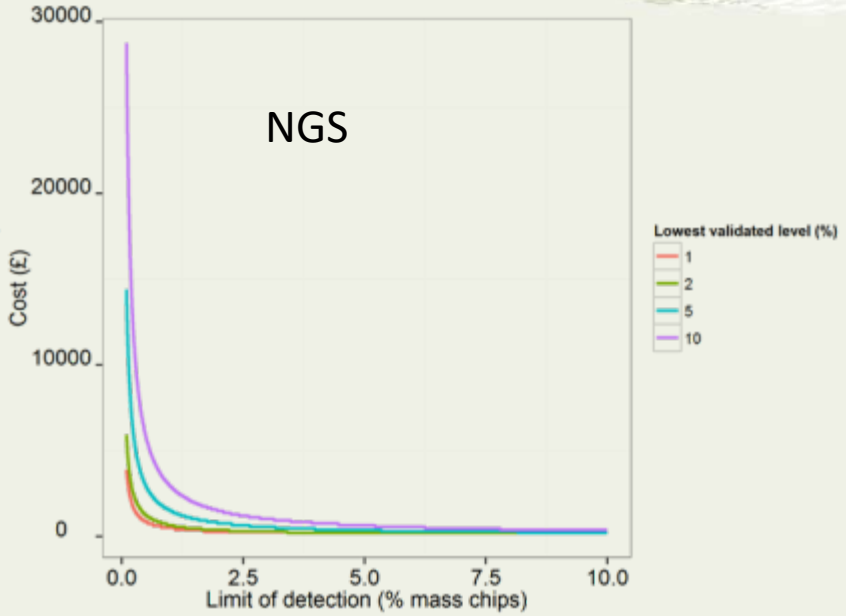
Target	ESTIMATES					VARIATION			Summary
	Mean proportion	mean(logit)	se mean	T2	se t2	DNA	Duplicate	Point	
Alnus	60.46%	0.42	0.114	0.010	0.064	0.511	0.000	0.000	High, homogenous, big analytical variation
Unassigned	23.34%	-1.19	0.112	0.119	0.072	0.485	0.000	0.000	High, homogenous, big analytical variation
Strobus	2.62%	-3.61	0.220	0.053	0.161	0.850	0.000	0.000	Homogenous, large analytical variation
Picea	2.03%	-3.88	0.173	0.358	0.191	0.295	0.175	0.000	fairly homogenous, small analytical variation
Quercus	0.90%	-4.70	0.266	-0.200	0.310	0.559	0.000	0.000	Low, homogenous, big analytical variation
Carya	0.84%	-4.77	0.306	-0.218	0.301	0.824	0.000	0.000	Low, homogenous, big analytical variation
Salix	0.73%	-4.92	0.251	0.128	0.341	0.000	0.000	0.000	Low, homogenous
Fraxinus	0.63%	-5.07	0.354	0.981	0.223	0.991	0.578	0.000	Low with single result at 50%.Variation between sampling times?
Pinus	0.36%	-5.61	0.354	-0.516	0.571	0.000	0.000	0.000	Low, homogenous
Acer	0.21%	-6.18	0.453	1.196	0.369	1.124	0.000	0.000	Low with few high results, variation between sampling times, very large analytical variation
Ulmus	0.06%	-7.50	1.234	-5.045	1.013	2.979	0.924	0.000	Low with few high results, variation between sampling times, very large analytical variation
Samubucus	0.05%	-7.70	1.000	1.749	1.081	0.000	0.000	0.000	Low, homogenous

Options for detecting wood genera





Target LOD (%)	Cost with Taqman (£)		Cost with sequencing (£)	
0.1	2000	2700	3800	34000
0.2	990	1400	1900	17000
0.5	410	570	860	6900
1	230	360	490	3500
2	140	210	250	1700
5	80	130	130	740
10	50	90	100	470

Confirming geographic origin?

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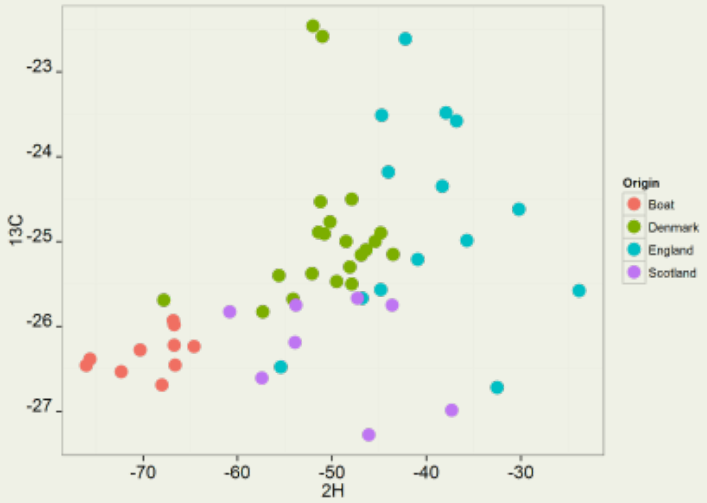

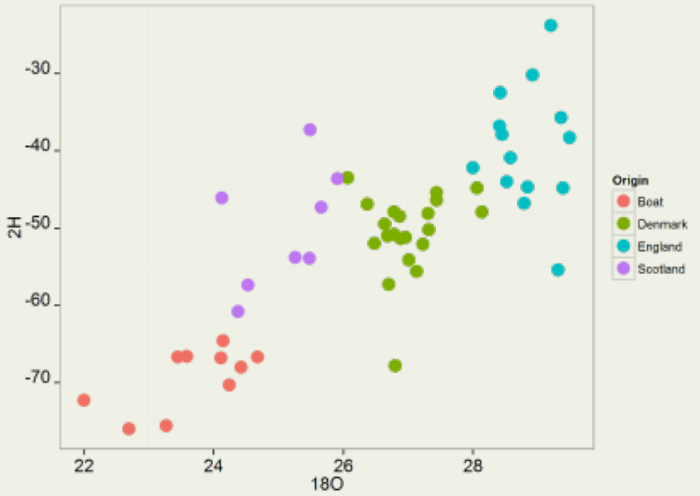


```
Random effects:
 Groups             Name        Variance Std.Dev.
 Origin:Site:Tree   (Intercept) 0.07570  0.2751
 Origin:Site        (Intercept) 0.04928  0.2220
 IRMSbatch          (Intercept) 0.13063  0.3614
 Residual                       0.05796  0.2407
Number of obs: 35, groups:  Origin:Site:Tree, 22;
Origin:Site, 11; IRMSbatch, 3

Fixed effects:
                Estimate Std. Error t value
(Intercept)      27.1120     0.2949   91.94
OriginEngland     1.7612     0.4863    3.62
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Conclusions

- Because we are interested in detecting tiny populations in large lots direct detection of pests and pathogens is a technical possibility, but very expensive to implement because of very large required sample sizes

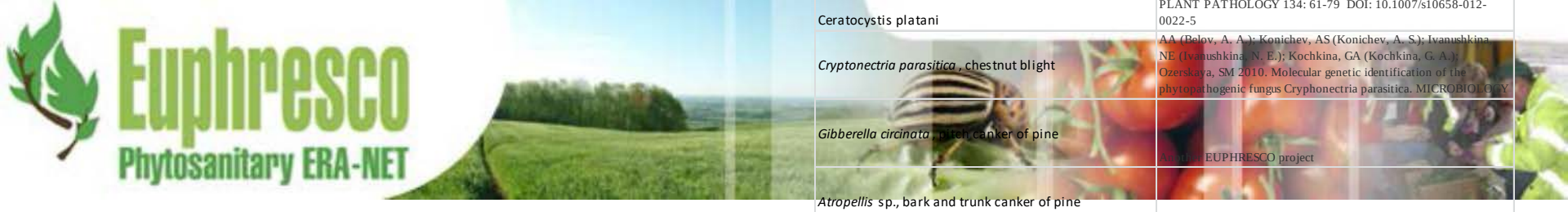
- There are other more practical options for reducing the risk associated with lots: confirming that host wood genera are absent, or present only at very low levels; confirming the geographical origin of lots.

- Both of these need further work to fully develop

Priority list of P&P

Insects	Host range	Natural distribution	Remarks	Pretreatments
Agrilus planipennis, Emerald Ash Borer	Most *Fraxinus* spp.	North-eastern China, Japan, Korea Republic, Mongolia, Russia (Far East) and Taiwan	Introduced to North East America 2002 where it has caused the death of millions of ash trees. No effective control methods are currently available.	Survival in wooden chips has been proven.
Agrilus anxius, Bronze Birch Borer	*Betula* spp.	North America	Main pathway could be wood chips from Canada and the USA. Probability of establishment in Europe is considered as to be "very high".	
Anoplophora glabripennis, Asian Long-horned Beetles	*Populus* spp., *Salix matsudana*, *Ulmus pumila*, *U. laeuig*, and *Acer* spp. Other species: *Aesculus chinensis*, *Alnus* spp., *Betula platyphylla*, *Elaeagnus angustifolia*, *Fraxinus* spp., *Hippophae rhamnoides* L. spp., *Malus sylvestris*, *Sinensis* (buckthorn), *Platanus orientalis,* and *Tilia tuan*	Japan, Korea and China	Introduced to North East America several times. Eradication campaigns for billons of $ have been carried out. Several times with success. Main pathway wooden packing material	ISPM 15 required for wood packaging material from place of origin. But this has proven not always to be efficient.
Anoplophora chinensis, Citrus Long-horned Beetle	Polyphagus on: *Acer, Citrus, Cryptomeria japonica, Malus, Populus, Salix, Ficus, Hibiscus, Mallotus, Platanus, Pyrus and Rosa.*	China, Hong Kong, Korea Republic, Malaysia, Myanmar and Vietnam.	Introduced to Europe several times. Main pathway living plants for planting or bonsai trees	
Xylosandrus crassiusculus, Asian ambrosia beetle	*Carya illinoinensis, Ceratonia siliqua, Diospyros kaki, Ficus carica, Malus domestica, Prunus avium, P. domestica, P. persica,*	Bhutan, China, India, Indonesia, Japan, Korea Malaysia, Myanmar, Nepal, Pakistan, Philippines, Sri Lanka, Taiwan, Thailand,		

Continued……



Insects	(q)PCR assay
Agrilus planipennis, Emerald Ash Borer	Developed assay in lab
Agrilus anxius, Bronze Birch Borer	Developed assay in lab
Anoplophora glabripennis, Asian Long-horned Beetles	Developed assay in lab
Anoplophora chinensis, Citrus Long-horned Beetle	Developed assay in lab
Xylosandrus crassiusculus, Asian ambrosia beetle	none found Perhaps X crassiusculus can be split into two species based on genbank sequences. Unsure to which type an assay should be designed
Xyleborus glabratus, Redbay ambrosia beetle	AAGTCAACTGAGGCTCCTTCGT TaqMan® probe: 311T: CACCGCTTGCCGCAAATATTGCC are specific to Xyleborus glabratus based on sequence alignments
Monochamus sutor, Pine sawyer	none found Very difficult to make general Monochamus assay as COI sequences are not very different from Anoplophora and others and they are also different within Monochamus. Not many ribosomal sequences.
Monochamus sartor,	none found
Monochamus galloprovincialis, Black pine sawyer	none found
Dryocosmus kuriphilus, Oriental chestnut gall wasp	detection of Dryocosmus kuriphilus Yasumatsu (Hymenoptera: Cynipidae) in chestnut dormant buds by nested PCR. BULLETIN OF ENTOMOLOGICAL RESEARCH 102: 367-371 DOI: 10.1017/S0007485311000812
Dendroctonus valens, Red turpentine beetle	DETECTION OF RED TURPENTINE BEETLE (DENDROCTONUS VALENS LECONTE) USING NESTED PCR. ENTOMOLOGICA AMERICANA 119: 7-13 DOI: 10.1664/11-RA-010R.1
Xylosandrus mutilatus, Camphor shoot beetle	none found
Polygraphus proximus, Sakhalin-fir bark beetle	none found
Gnathotrichus materiarius, hickory borer	none found
Ceratocystis platani	PLANT PATHOLOGY 134: 61-79 DOI: 10.1007/s10658-012-0022-5
Cryptonectria parasitica, chestnut blight	AA (Belov, A. A.); Konichev, AS (Konichev, A. S.); Ivanushkina, NE (Ivanushkina, N. E.); Kochkina, GA (Kochkina, G. A.); Ozerskaya, SM 2010. Molecular genetic identification of the phytopathogenic fungus Cryphonectria parasitica. MICROBIOLOGY
Gibberella circinata, pitch canker of pine	Another EUPHRESCO project
Atropellis sp., bark and trunk canker of pine	none found
Mycosphaerella populorum, Septoria canker of poplar (though less likely in wood chips as a leaf pathogen)	sensitive PCR-based detection of Septoria musiva, S-populicola and S-populi, the causes of leaf spot and stem canker on poplars. MYCOLOGICAL RESEARCH 109: 1015-1028 DOI: 10.1017/S0953756205003242
Dothistroma pini, red band needle blight (NA type), (needle pathogen as above)	Development, Comparison, and Validation of Real-Time and Conventional PCR Tools for the Detection of the Fungal Pathogens Causing Brown Spot and Red Band Needle Blights of Pine. PHYTOPATHOLOGY 100: 105-114 DOI: 10.1094/PHYTO-100-1-
Cronartium quercuum (f.s. fusiforme), fusiform rust of pine (or *Cronartium* sp.)	none found
Inonotus weirii, laminated root rot	based method for the identification of important wood rotting fungal taxa within Ganoderma, Inonotus s.l. and Phellinus s.l. FEMS MICROBIOLOGY LETTERS 282: 228-237 DOI: 10.1111/j.1574-6968.2008.01132.x
Hymenoscyphus pseudoalbidus (*Chalara fraxinea*), ash dieback	Chandelier, A; Andre, F; Laurent, F 2010 Detection of Chalara fraxinea in common ash (Fraxinus excelsior) using real time PCR. FOREST PATHOLOGY 40: 87-95 DOI: 10.1111/j.1439-0329.2009.00610.x
Lecanosticta acicola, brown spot needle blight (though less likely in wood chips as a needle pathogen) (syn Mycosphaerella dearnessii)	Development, Comparison, and Validation of Real-Time and Conventional PCR Tools for the Detection of the Fungal Pathogens Causing Brown Spot and Red Band Needle Blights of Pine. PHYTOPATHOLOGY 100: 105-114 DOI: 10.1094/PHYTO-100-1-
Botryosphaeria laricina (syn. *Guignardia laricina*), shoot blight of larch	none found
Anisogramma anomala, Eastern filbert blight on hazelnut	Molnar, TJ; Walsh, E; Capik, JM; Sathuvalli, V; Mehlenbacher, SA; Rossman, AY; Zhang, N. 2013. A Real-Time PCR Assay for Early Detection of Eastern Filbert Blight. PLANT DISEASE 97: 813-818 DOI: 10.1094/PDIS-11-12-1041-RE
Oomycetes	
P. kernoviae	Boonham, N; Lane, CR 2011 Development of a real-time PCR assay for detection of Phytophthora kernoviae and comparison of this method with a conventional culturing technique. EUROPEAN JOURNAL OF PLANT PATHOLOGY 131: 695-703 DOI:
Phytophthora ramorum	2009 Multiplex real-time polymerase chain reaction (PCR) for detection of Phytophthora ramorum, the causal agent of sudden oak death. CANADIAN JOURNAL OF PLANT PATHOLOGY 31: 195-210
Bacteria	
Erwinia amylovora, fireblight	Dreo, T; Pirc, M; Ravnikar, M 2012. Real-time PCR, a method fit for detection and quantification of Erwinia amylovora. TREES-STRUCTURE AND FUNCTION 26: 165-178 DOI: 10.1007/s00468-011-0654-7
Pseudomonas syringae pv. *aesculi*, bleeding canker of horse chestnut	Infection of horse chestnut (Aesculus hippocastanum) by Pseudomonas syringae pv. aesculi and its detection by quantitative real-time PCR. PLANT PATHOLOGY 58: 731-744 DOI:

‘Condensed’ list

P&P:

- *Hymenoscyphus pseudoalbidus* (*Chalara fraxinea*), ash dieback
- Asian Long-horned Beetles (*Anoplophora glabripennis*)
- Citrus Long-horned Beetle (*Anoplophora chinensis*)
- Emerald Ash borer (*Agrilus planipennis)*
- *Phytophthora ramorum* (DNA only)
- *Phytophthora kernoviae* (DNA only)

SAMPLE PREPARATION

Commercial samples:
Pine bark

Procedure:
Grinding in a mortar with N2 or cut to pieces

DNA EXTRACTION

Woodchip, either ground(by mortar and N2) or cut to pieces by hand

2 grs to 10 grs

1. Put material to analyse into a flask of convenient size (250-400 ml)
2. Add 100 ml extraction buffer per 10 grs material + 2% W/V polyvinilpolipyrrolidone (PVPP)
3. Shake at room temperature at 250 rpm for 30 min
4. Leave on bench to settle for 10 min
5. Decant into falcon tubes (50 ml) filtering through whatmann paper (put a small amount to filter and change the paper if necessary due to clogging until all liquid is placed in 1 or 2 falcon tubes
6. 10.000 rpm 5 min to pellet the debris and residues of wood
7. Decant the liquid into new falcon tubes. add 0.6 V/V isopropanol to each. Mix inverting the tubes
8. 10.000 rpm 10 min. Eliminate supernatant
9. Dry on bench (about 1 h). Normally still there will be a brown pellet
10. Resuspend each tube in 500-100 µl water. Vortex. Probably not all pellet will be suspended
11. (optional): Mix all suspensions from the same sample into an eppendorf tube. Centrifuge at 13.000 rpm 5 min to pellet more debris
12. Take supernatant (probably still brownish) into a 2 ml eppendorf
13. Purification step using the Plant DNeasy mini kit as follows:
14. Add to the suspensions 3 vol of buffer AW1, mix. Pass through Qiashreded column and centrifuge at 8000 rpm 1 min
15. Recover supernatant and pass all the volume through DNeasy column
16. Wash 2X with 500µl buffer AW2
17. Add 100 µl water and recover the DNA.

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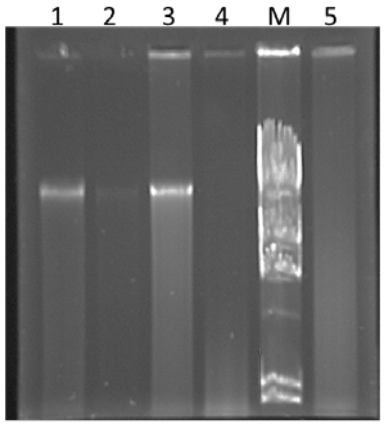
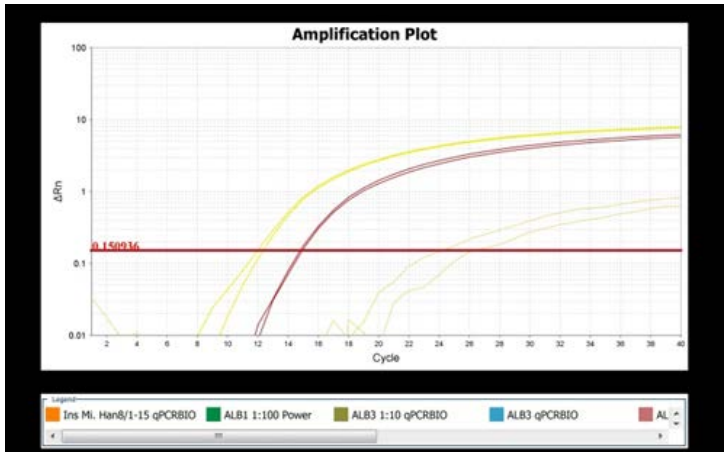
RESULTS OBTAINED WITH THE EXTRACTION PROTOCOLS



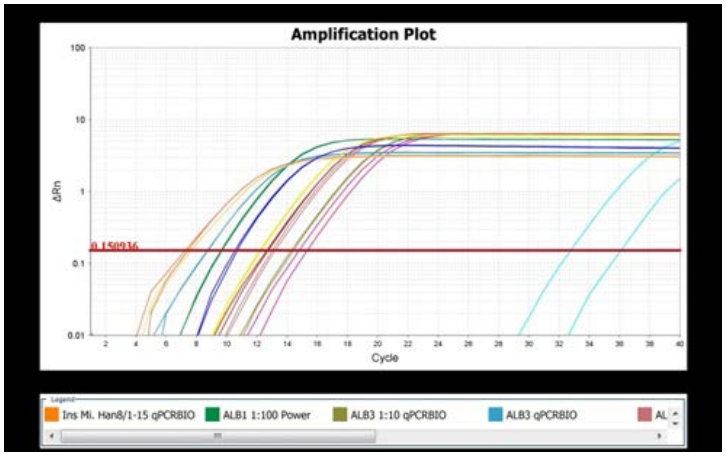
Sample	Ct
1 DNA not purified	0
1 DNA not purified	0
1 DNA not purified 1/10	37.9
1 DNA not purified 1/10	37.3
2 DNA purified Qiagen	35.8
2 DNA purified Qiagen	35.7
2 DNA purified Qiagen 1/10	33.1
2 DNA purified Qiagen 1/10	33.5
3 DNA Qiashredded + Qiagen	0
3 DNA Qiashredded + Qiagen	0
3 DNA Qiashredded+ Qiagen 1/10	0
3 DNA Qiashredded+ Qiagen 1/10	0
4 DNA + phenol	0
4 DNA + phenol	0
4 DNA + phenol 1/10	36.7
4 DNA + phenol 1/10	35.6
5 DNA + Powersoil	33.8
5 DNA + Powersoil	32.8
5 DNA + Powersoil 1/10	36.6
5 DNA + Powersoil 1/10	36.0
C-	0
C+	29.1

Figure 1. Gel at 0,6% with different extraction methods with ground samples with mortar and N2. Lanes: 1 USDA method (1 g sample), 2: Sample with Llop et al method + phenol purification (5 g); 3: DNA suspension with Llop et al method + column of plant easy mini kit (Qiagen); 4: sample with Llop et al method + pvpp added and no purification steps; M: HindIII Lambda marker; 5: DNA suspension without mini kit purification.

Specific tests on Agrilus and Anoplophora



Agrilus



Anoplophora

Detection of *Hymenoscyphus pseudoalbidus* after dacay

- The woodchip mixtures were constructed with decreasing amounts of *Quercus*, *Pinus* and *Fraxinus*: 10%, 1.0% and 0.1%. The remaining 70-99.7% was *Populus*.
- The woodchip mixtures were inoculated by 10, 3, and 1 g of infected *F. excelsior* L. in the respective 10%, 1.0% and 0.1% composition.
- The woodchip mixtures were treated with 5 treatments viz., Heat treatment 1 (56^0 C for 30 min), Heat treatment 2 (75^0 C for 30 min), Heat treatment 3 (100^0 C for 30 min), Decay treatment 1 (2 weeks at 30^0 C and 90% MC), and Decay treatment 2 (3 weeks at 30^0 C and 90% MC).
- After treatments, the woodchip mixtures were crushed using a cutting mill (Retsch SM2000, Germany), and DNA was extracted from 5, 15 and 25 g of crushed woodchip mixtures.
- DNA was diluted into 100-fold.
- qPCR were performed using real-time PCR primers and TaqMan probe according to loos *et al*. 2009 (Eur. J. Plant Pathol. 125: 329-335).

Results

Table: Detection of *Hymenoscyphus pseudoalbidus* using TaqMan qPCR assay

Treatments	**10% contamination**			**1.0% contamination**			**0.1% contamination**			**Control (no contamination)**		
Sub-samples	5g	15g	25g	5g	15g	25g	5g	15g	25g	5g	15g	25g
Heat treatment -1	30.84± 0.04	30.90± 0.13	30.93± 0.09	37.33± 0.58	32.60± 0.09	34.90± 0.30	29.47± 0.28	32.17± 0.09	30.72± 0.01	0±0	0±0	37.63[b]
Heat treatment -2	33.06± 0.81	36.99± 0.51	35.55± 0.55	34.96± 0.28	0±0[a]	34.52± 1.12	35.01± 0.79	33.60± 0.16	37.32± 2.33	0±0	0±0	0±0
Heat treatment -3	31.67± 0.11	32.18± 0.01	31.86± 0.35	32.39± 0.13	32.11± 0.59	31.79± 0.34	31.84± 0.27	31.56± 0.28	32.70± 0.08	38.47[b]	0±0	0±0
Decay treatment -1	40.98± 1.99	30.05± 0.40	29.69± 0.06	32.75± 0.36	33.85± 0.30	35.96± 0.76	31.66± 0.16	30.49± 0.05	31.90± 0.11	0±0	0±0	0±0
Decay treatment -2	36.38± 0.10	32.03[b]	32.24±0	32.58± 0.42	31.31± 0.07	33.41± 0.01	32.12± 0.13	30.72± 0.79	31.95± 0.40	0±0	0±0	0±0
No treatment (control)	29.86± 0.52	31.06± 0.01	30.95± 0.08	40.18± 4.07	33.33± 0.20	36.52± 0.01	33.47± 0.17	31.63± 0.21	30.52± 0.09	0±0	0±0	0±0

Data are mean C_T value ± SD standard deviation of two technical replicates except from the red mark, and 0 indicates no C_T value.

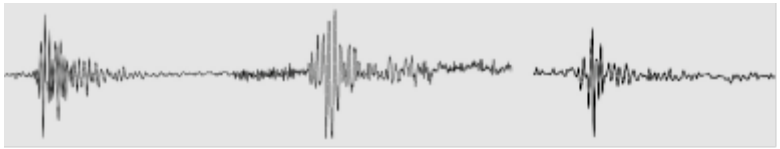
[a]The C_T value could be false negative as it was amplified in 10x diluted DNA.

[b]The C_T value could be false positive because it was obtained from one well, the other well was zero (0).

HUGE AMOUNTS OF SAMPLE TO BE PROCESSED AND ANALYSED – ALTERNATIVES?

- NEW TECHNOLOGIES THAT ALLOW IN SITU ANALYSIS
- LARGE AMOUNT OF SAMPLE ANALYSED
- NO PROCESSING OF SAMPLES
- SIMPLE SAMPLING METHODOLOGY

Laser vibrometry system for diagnosis of insects in wood and crops (Sanders et al, 2011; Zorovic and Cokl, 2014)
Electronic noses to detect fungi in wood (Casalinuovo et al, 2006; Baietto et al, 2010; Fiers et al, 2013)
Hyperspectral imaging for diagnosis of nematodes (Sivertsen et al, 2012) and magnetic resonance imaging for detection of fungal wood decay, (Muller et al, 2002)



Targeted sampling
- Trapping (insects)
- Air (fungal spores)

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